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(54) Title: TRANSGENIC WHEAT

(57) Abstract

A novel method for producing transgenic wheat plants, vectors for use in the method and novel transgenic wheat plants.

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TRANSGENIC WHEAT

FIELD OF THE INVENTION

The present invention provides a method for producing transgenic wheat plants which comprises introducing foreign DNA via the pollen tubes, thereby obtaining a transgenic embryo which can develop into a mature transgenic plant.

The present invention also provides transgenic wheat plants.

BACKGROUND OF THE INVENTION AND PRIOR ART

Since the advent of recombinant DNA technology and its successful application in the transformation of various organisms, one of the main goals has been to apply this technology for the transformation of plants in general and agriculturally important crops, e.g. wheat, in particular. Such transformation aims at the production of transgenic plants having new traits which provide certain growth advantages such as improved root systems, herbicide resistance and pest resistance.

With a steadily increasing world population it has long been realised that there exists an urgent need for increasing the yield of important crops such as grain crops, in particular wheat, and this by developing new varieties of such plants that have the aforesaid growth advantages.

Various methods for transforming plants have been developed. One such method involves the preparation of protoplasts from plant tissue, the transformation thereof *in vitro* and subsequently the regeneration of the transformed protoplasts into transgenic whole plants.

Other methods employ the *Agrobacterium tumefasciens* system by which recombinant T-DNA from Ti or Ri plasmids become inserted into the nuclear DNA of plant cells. Yet another method for transforming plant tissues with foreign DNA involves the bombardment of plant cells with DNA-coated carrier particle projectiles, whereby some of these projectiles penetrate the plant cells and insert the foreign DNA therein.

Various other methods of micro-injection of foreign DNA into plant organs, in particular reproductive or regenerative organs have also been employed for the purposes of producing transgenic plants.

All of the aforesaid methods were developed primarily for the transformation of *Dicotyledonous* plants and were successfully applied in such plants. However, they were found to be less applicable for transforming *Monocotyledonous* plants, in particular wheat plants.

One of the reasons that the aforesaid methods were not applicable to many *Monocotyledonous* plants is the fact that it is very difficult to regenerate their cells grown in tissue culture into whole plants.

A method for transforming wheat plants which involves plasmid DNA uptake during pollen tube development was reported by Picard et al., 1988 in "Proceedings of the Seventh International Wheat Genetics Symposium, Vol. 1, p.779-781", Miller and Koebner Eds., Cambridge, England, 13-19 July, 1988. These authors reported that they used plasmids

containing the aminoglycoside phosphotransferase II gene (NPTII) which confers kanamycin resistance when expressed, and plasmids containing the NPTII gene and putative wheat chromasomal origins of replication for enhancing foreign DNA integration. The plasmids were introduced into exposed pollen tubes by first removing the tips of pollinated stigmas in which the pollen tubes were growing to expose these tubes and then adding thereto a solution containing the plasmids. The plasmid DNA apparently migrated down the growing pollen tubes together with the male nuclei and so entered the ovules during fertilization. As a result, about 1% of the seeds which were obtained were resistant to kanamycin as demonstrated by their ability to germinate and grow on media containing kanamycin and by the ability of such germinating plants to grow into mature wheat plants in soil always soaked with water containing 100 mg/l kanamycin. Southern blot analysis with an NPTII gene probe further revealed that DNA extracted from the kanamycin resistant plants contain the NPTII sequence with plasmids containing the above-noted wheat origins of replication being the most frequent. Furthermore, when these plants were self-crossed the resulting second generation of plants (F_2) were all kanamycin resistant which indicates that the NPTII sequences were transferred to the second generation. No distinction between true integration into the wheat genome and putative NPTII-carrying endophytes was presented.

However, it must be pointed out that since the aforesaid publication there have been no subsequent publications confirming the claimed success of this transformation procedure in wheat. It should be noted that various publications and conferences subsequent to the above publication revealed that the workers in the field have, in fact, not been able to repeat the above procedure and have reverted to the various other techniques mentioned hereinabove for transforming wheat. The aforesaid techniques were addressed in a recent review of current procedures for

transforming cereal plants, including wheat (Potrykus, 1990, Bio/Technology 8, 535–542), in which it was concluded that while the pollen tube procedure should be investigated further, it did not hold much promise for success; in the author's words "Pollen Tube Pathway: No transgenic plants have been recovered; probably not much potential".

It should also be noted that the aforesaid procedure has since been carried out with success only in rice plants (Luo and Wu, 1989, Plant Molecular Biology Reporter 7, 69–77), which although being monocotyledonous differ in many physiological respects from wheat.

Furthermore, it should also be noted that in all of the reported transformed wheat plants, by way of any of the above methods, none of the wheat strains were commercial crop cultivars, i.e. agricultural cultivars grown on a large scale in order to obtain wheat crops. Many of the putative transformed wheat plants would have little agricultural application and, at best, could be used as a starting point to try and obtain a transformed commercial wheat strain, e.g. by back–crossing and by crossing with other varieties.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide a method for producing transgenic wheat plants in particular transgenic wheat plants of a commercial crop variety, i.e. one of many cultivated varieties used throughout the world for growing wheat crops.

It is a further object of the present invention to provide a transgenic wheat plant of a commercial variety.

A still further object of the present invention is to provide the appropriate recombinant DNA molecules for the purposes of producing the aforesaid transgenic wheat plants.

All other objects of the invention will be revealed in the following description and claims.

In accordance with the above-noted objects of the present invention, there is provided a method for producing transgenic wheat plants comprising the steps of:

(a) emasculating wheat plant florets by removing all pollinator anthers prior to their maturation and then inducing synchronized pollination by pollinating the stigmas of said florets with mature male pollinator anthers from the same or a different variety of wheat plant;

(b) applying a droplet of an aqueous DNA solution onto one or more pollinated stigmas, following a time period after pollination in which the pollen tubes in the pollinated stigmas have started to grow into the style but have not yet reached the ovary, said DNA solution comprising a DNA vector carrying at least one gene foreign to the plant and capable of inducing the expression of a desirable trait in the plant and optionally an additional marker gene;

(c) maintaining said DNA solution droplet on said stigma in a humid environment for a period of time to ensure that said DNA vector reaches and enters the ovule;

(d) protecting the treated plants of step (c) from additional pollination by any nearby plants, growing said plants and collecting the seeds developed in said florets; and

(e) growing said seeds under conditions adapted for the selection of transformed wheat plants.

In accordance with one embodiment of the invention, to be referred to herein at times as the *"truncation embodiment"*, the stigmas are truncated after pollination and the droplet of an aqueous DNA solution is applied onto the truncated stigmas. In accordance with another embodiment of the invention, to be referred to herein at times as the *"intact stigma*

embodiment", the droplet of an aqueous DNA solution is applied onto intact stigmas following a time period after pollination.

Preferably, the aforesaid truncation is carried out by truncating the stigma tips, about ½–2½, more preferably about 1–2 hours after pollination. In the case of the spring–bread (*T. aestivum L.*) cultivars such as cultivars ATIR (CNO "S"/PJ62/4/GLL/3/TOB//JAR "S"/CRESPO, Hazera Seed Co., Israel), BAGULA "S" (CM 59123, CIMMYT, Mexico), CIANO 79 (CM31678, CIMMYT, Mexico), Genaro 81 (VEERY#3"S", CIMMYT, Mexico), KAUZ "S" (CM67458, CIMMYT, Mexico), KEA"S" (CM21335, CIMMYT, Mexico), MILAN (23IBWSN37, CIMMYT, Mexico), OPATA M 85 (CM40038, CIMMYT, Mexico), Papago M86 (CM52359, CIMMYT, Mexico), PAVON 76 (CM8399, CIMMYT, Mexico), Seri 82 (VEERY#5"S", CIMMYT, Mexico), and Shafir (SON64A/TZPP//NAI60/3/FA, Hazera Seed Co., Israel), all of which were tested in accordance with the invention, the truncation is preferably performed about 1½ hours after pollination. It is believed that this preferred time period between pollination and truncation would also apply to other wheat species and cultivars.

Generally, when performing the truncation embodiment of the invention, the interval between pollination and truncation of the stigma tips should be chosen in accordance with the wheat genotype used and the environmental conditions. Where, for example, the type of wheat cultivar and the environmental conditions favour relatively rapid pollen attachment to the stigmas and subsequent pollen tube development and growth within the stigmas and into the styles, this interval may be in the range of 1–1½ hours. Where, as another example, the type of wheat cultivar and the environmental conditions favour a slower attachment of the pollen and subsequent pollen tube development, the interval may be in the range of 1½–2 hours.

The above described timing between pollination and DNA application should also be adhered to when performing the intact stigma embodiment of the invention, contingent, as noted above, with the wheat genotype used, with the environmental conditions etc.

In accordance with both embodiments, the droplet of DNA solution applied in step (b) of the method is suitably maintained on the pollinated stigma (step (c)) for a time period sufficient to ensure that the DNA vector transported by the growing pollen tube reaches the ovule. A suitable time period was found to be at least 12 hours, e.g. for about 12–14 hours.

Also provided by the invention is a recombinant DNA vector for use in the above method.

The DNA vector of the invention comprises a gene which can induce in the plant the expression of a trait, the transfer of which into the plant is desired. Such a gene may for example be one expressing a nitrogen fixation structural protein or one expressing a protein that confers herbicide resistance to the plant. Additionally, the vector may also suitably comprise a reporter gene, e.g. the *uid*A gene encoding *E. coli* β–glucuronidase, which serves as an indicator of successful transformation: cells expressing this gene when provided with the substrate (X–Gluc) for the expressed enzyme are capable of converting this susbstrate into a detectable product.

The DNA vector preferably comprises also a plant selectable marker gene which may, for example, be one encoding an antibiotic resistance, e.g. kanamycin resistance. A non–limiting example of such a gene, used in the experiments conducted within the framework of the present invention, is the APH(3')II (NPTII) gene linked to the nopaline synthase (NOS) promoter and to the polyadenylation signal sequence of the T_L–DNA (gene 4) (Gielen, J. et al., 1984, Koncz et al., 1989). The APH(3')II (NPTII)

gene encodes the enzyme aminoglycoside phosphotransferase and thus confers kanamycin resistance to the transformed plants.

Another bacterial selectable marker gene e.g. Amp^R may also be included in the vector for the purpose of propagating the DNA vector in bacteria in order to obtain large quantities thereof needed for the preparation of said DNA solution.

Non-limiting examples of such DNA vectors, used in the experiments conducted within the framework of the present invention, are the plasmids pPCV702*nif*D and pPCV702GUS, both of which comprise the afore-mentioned APH(3')II gene and, in pPCV702*nif*D, the *Klebsiella pneumoniae nif*D coding region linked to a CaMV 35S promoter and a NOS terminator and, in pPCV702GUS, the coding region of β-glucuronidase (GUS) also linked to a CaMV 35S promoter and a NOS terminator. Accordingly, wheat plants transformed with either of these plasmids, by the method of the present invention, are kanamycin resistant and/or capable of expressing the GUS enzyme.

The invention also provides transgenic wheat plants of agronomic, commercial crop varieties.

In accordance with the present invention, a long-felt need is fulfilled whereby transgenic wheat plants of several commercial varieties, have been obtained for the first time. Transgenic wheat plants obtained in accordance with the invention were grown through 5 generations without a loss of the foreign gene with which the original plants were transformed.

BRIEF DESCRIPTION OF THE DRAWINGS

The figures in the annexed drawings illustrate the following:

Fig. 1 is a schematic representation of the plasmid pPCV702*nif*D;

Fig. 2 is a schematic representation of the plasmid pPCV702GUS;

Fig. 3a shows results of Southern blot hybridization of a NPTII gene probe to DNA samples, digested with *BamHI* and *HindIII* restriction endonucleases, from wild type wheat varieties; plants transformed according to the procedure of the present invention and from the plasmid pPCV702*nif*D, described in Example 4;

Fig. 3b is a schematic restriction map illustrating the pertinent restriction endonuclease sites of pPCV702*nif*D integrated into the wheat genome in the transformant designated TAU89107-1, as determined from Southern blot results (Fig. 3a, Fig. 4a and Fig. 5), as described in Example 4;

Fig. 4a shows results of Southern blot-hybridization of a NPT II probe to DNA samples, digested with PstI restriction endonuclease, from wild type wheat varieties and plants transformed with pPCV702*nif*D according to the present invention, as described in Example 4;

Fig. 4b is a schematic restriction map illustrating the pertinent restriction endonuclease sites for pPCV702*nif*D integrated into the wheat genome as determined from the Southern blot results (Figs. 3a, 4a and Fig. 5), as described in Example 4;

Fig. 5 shows results of Southern blot-hybridization of a NPT II fragment and the rest of the 702*nif*D vector with DNA samples digested with Bam HI and Pst I from a wild-type wheat plant and second generation (F_2) transformed wheat plants obtained by selfing of first generation plants transformed by plasmid pPCV702*nif*D, as described in Example 4;

Fig. 6 shows the results of Southern blot hybridization of a NPTII fragment with BamHI and HindIII digested DNA sample of the second generation (F_2) of transformed wheat plants, obtained by selfing of the transformant TAU89107-1, as described in Examples 4 and 5;

Fig. 7 shows the results of Southern blot hybridization of a NPIII fragment with BamHI and HindIII digested DNA samples of the third

generation (F_3) of transformed wheat plants obtained by selfing of plants from the TAU89107 F_2 families, as described in Examples 4 and 6;

Fig. 8a shows the results of an agarose gel electrophoresis of DNA samples obtained by PCR amplification of the APH(3')II gene present in the transformed wheat plants of the Shafir cultivar. DNA samples were extracted from F_2, F_3 and F_4 plants originating from the transformant TAU89107-1 and resulting from self pollination of each generation of plants, as described in Example 7;

Fig. 8b shows the results of an agarose gel electrophoresis of DNA samples obtained by PCR amplification of the APH(3')II gene and its gene 4 terminator, using total DNA extracted from the F_4 progenies of transformed TAU89-107-1 obtained by selfing, as described in Example 7;

Fig. 9 shows the results of Southern blot hybridization of a gene 4 terminator fragment with DNA samples of a wild type Shafir wheat plant and the F_4 generation progenies of the transformant TAU89107-1. The DNA samples were digested with BamHI and HindIII. All four generations resulted from self-pollination, as described in Examples 4 and 8;

Fig. 10a shows the F_5 progeny CC44 (TAU89107-1-J18-9-1B-4A) of the TAU89107-1 transformant which produced a normal shoot upon growing on kanamycin containing medium as compared to the wild type Shafir plant which turned white under the same growth conditions, as described in Example 9;

Fig. 10b demonstrates gel separation analysis of the PCR amplification products of the NPTII tail (51bp) and its gene 4 terminator using DNA templates extracted from CC44 transformant or from plants of the wheat cultivar Shafir, as described in Example 9;

Fig. 11 shows the results of Southern-blot hybridizations of the first generation (F_1) of wheat plants of the commercial cultivars Pavon 76 and Seri 82 transformed with pPCV702GUS and probed with the same plasmid,

the DNA samples having been digested with EcoRI and HindIII, as described in Example 10;

Fig. 12 shows the results of an agarose gel electrophoresis of DNA samples obtained from PCR amplification of the APH(3')II and GUS genes from DNA extracts from F_1 transformants of Seri 82 cultivar, as described in Example 10; and

Fig. 13 shows a representation of the electrophoresis on an agarose gel of the 0.6 kb PCR product obtained from F_1 generation transformants, transformed with the plasmid pPAT-NPTII 100.1-28 carrying the *Streptomyces hygroscopicus bar* gene, as described in Example 12.

DESCRIPTION OF SPECIFIC EMBODIMENTS

The present invention will now be described in more detail in the following examples and the annexed Figures. These examples should be construed as an illustration of the present invention and should not be considered limiting.

Example 1: Plasmids

Two plasmids, pPCV702*nif*D and pPCV702GUS, were constructed as illustrated in Figs. 1 and 2, respectively, both derived from the vector pPCV702 (Koncz and Schell, 1986; and Koncz et al., 1989). In both plasmids, the APH(3')II or NPT II gene encoding aminoglycoside (kanamycin) phosphotransferase, linked to the NOS (nopaline synthase) promoter and to the TL-DNA *ipt* gene (gene 4) polyadenylation signal, or PolyA, (terminator), served as a selective marker. This gene, when expressed in plants, confers kanamycin resistance.

The plasmid pPCV702*nif*D contains the coding region of *Klebsiella pneumoniae nif*D, situated between the CaMV 35S promoter and NOS terminator.

The plasmid pPCV702GUS includes the coding region of the β-glucuronidase (GUS) gene, a reporter gene, linked to the CaMV 35S promoter and to the NOS terminator.

A mixture of supercoiled and relaxed (nicked) forms of the aforesaid plasmid DNA, dissolved in 10 mM Tris, 1 mM EDTA, pH 8, was used for preparing the DNA solutions to be applied onto the stigmas in the transformation methods described hereinbelow.

Example 2: Preparation of Wheat Plants and Administration of DNA

Wheat plants selected from several commercial wild spring bread wheat cultivars, were grown in pots in a temperature-controlled greenhouse (21 ± 2°C) with supplementary lights. In addition, a second group of wheat plants, of the same above variety, were grown in a nethouse in sandy-loam soil. These groups of wheat plants were sown at the start of the experiments, with additional sowings repeated at 10-day intervals to ensure constant supply of pollen when needed for pollination. When wheat plants reached growth state 57 (3/4 of ear emerged) the spikes of the experimental plants were treated as follows: each spikelet was cut at half its height, the middle florets removed, leaving the two outer florets. The immature stamens were removed from each of the two remaining florets (per spikelet). All spikelets on one side of the rachis were marked at their base with a water-proof marker. Thereafter, the spike was covered with a glassine paper bag.

When the stigma of each of the two floret pistil of the median spikelets on the aforesaid treated spike started becoming feathery and spread outward (ca. 4-5 days after emasculation), i.e. exhibited signs of pistil maturity, the glassine paper bag was cut across the top and a male pollinator spike (of same or different cultivar) with its spikelets cut in half, to allow mature stamens to protrude outward from the glumes, was inserted into the

bag. The pollinator spike was left inside the bag for 20–30 min. to allow its stamens to burst and release their pollen. The pollinator spike was swirled around several times to allow pollen to reach all florets of the spike. Thereafter, the pollinator spike was removed and the glassine paper bag sealed with a clip.

Between half an hour to two and a half hrs. later, usually one and a half to two and a half hrs. later, at the stage when the pollen tubes have formed and have begun growing within the pollinated stigmas (but have not yet traversed the styles to reach the ovary), the tip of the feathery stigmas was clipped with sharp, narrow–tipped scissors to expose the aforesaid pollen tubes and a 10 µl drop of a DNA solution (2–3 µg of plasmid DNA –Example 1) was placed on top of the cut stigmas of florets on the previously marked half spike with the aid of a micro–tip applicator. Florets on the non–marked half spike which served as control, received 10 µl of water. It should be noted that the aforesaid pollination procedure provides a synchronized initiation of pollen tube formation within the stigmas. Further, the aforesaid period between pollination and clipping of the pollinated stigma tips varies according to environmental conditions and the strain of wheat plants used, such that a period of about 1–1½ hrs is optimal for situations favouring relatively rapid pollination attachment and subsequent development of pollen tubes within the stigmas and into the styles, a period of 1½–2 hrs being optimal in situations where the aforesaid pollen attachment and pollen tube development is slower.

Treated, open–bagged plants with glassine bags pulled down and spikes fully exposed, were placed in a humidity chamber in the temperature–controlled greenhouse, where a water–mister provided fine mist for between 12 and 14 hr. Plants grown in the nethouse were treated in the afternoon when temperatures dropped to less than 20°C. Following introduction of DNA, plants were covered overnight, to allow growth of pollen

tubes to the ovaries and subsequent fertilization of the ova, with a plastic bag to supply condensed moisture, or were subjected to dew when appropriate. Following exposure to mist or dew, the glassine bags were pulled upward to cover treated spikes and sealed with paper clips. Detailed operational procedures were recorded for each treated spike.

Similar experiments were also carried out according to the above noted procedure but wherein the stigma tips were not cut, the DNA solution being added to the top of pollinated uncut stigmas. The results of both sets of experiments are set forth in Table 1 as referred to in Example 3 below.

Example 3: Bioassays

(a) APH(3')II Expression:

Seeds obtained by the transformation procedure of Example 2 using the pPCV702*nif*D vector were immersed in hypochlorite for 5 minutes, washed in distilled water and then transferred to water–agar petri dishes with 150 μg/ml kanamycin and placed in a growth chamber at 25°C. Ten days later, all seeds were transferred to MS medium and thereafter scored for the appearance of bleaching of the shoot and the number and length of roots recorded. In some experiments the seedlings were kept in the growth chamber for 4 days on a kanamycin containing medium and thereafter transferred to MS growth medium for a further 4 days and scored as noted above.

Seeds obtained from the above transformation procedure, representing the F_1 generation, were treated with hypochlorite and placed in water–agar petri dishes containing 150 μg/ml kanamycin, along with controls of parents without kanamycin. Germinating seeds with normal roots and unbleached primary leaves were transferred to pots and placed at 18°C (14 hr light/10 hr dark). These F_1 seedlings were grown to maturity and

seeds obtained therefrom, representing the F_2 generation, were then sown without selectable pressure for seed increase. Seeds obtained therefrom represent the F_3 generation.

To examine APTII activity, i.e. expression of the NPTII gene carried by the vectors used to transform the original parent plants, seedlings of the F_3 generation were germinated on water-agar containing 150 µg/ml kanamycin. The development of green leaves as compared to the white leaves of the untransformed controls, indicated that the APTII gene is present and expressed. These green seedlings were transferred to the greenhouse and their leaf DNA was analyzed by Southern blotting and PCR amplification of specific sequences (See Examples 4 to 11 below).

The above kanamycin selection procedure has been performed in all consecutive generations of the transformed plant line termed TAU89107-1, up to and including the F_5 (fifth) generation, this further indicating that the above transformation is stable.

(b) GUS Expression:

The expression of β-glucuronidase in roots excised from the axenically grown (i.e. isolated) F_1 seedlings, from the originally transformed plants transformed with pPCV702GUS, was demonstrated using X-Gluc as a substrate. The appearance of a blue color which resulted from a specific breakage of the 1-4 β bond of X-Gluc, indicated the activity of the introduced GUS in the roots (Jefferson et al., 1987). In 40% of the plants expressing GUS activity (Table 1) it was possible to detect the corresponding foreign gene (*uid*A so-called GUS) integrated into the genome by Southern analysis (see below in Example 10 and Figure 11). As noted above in Example 2, in this GUS assay a comparison was also made between F_1 seedlings originating from plants transformed by a DNA solution containing pPCV702GUS applied to cut stigmas (i.e. without the tips of the

stigmas) or applied to uncut stigmas. Surprisingly, the "uncut stigma" group gave better results than the "cut stigma" group (14.6% vs 5.8%), this result being in contrast to the previously held belief that it is necessary to physically expose the growing pollen tubes to the DNA solution (see references in the 'Background' section above).

TABLE 1

Estimation of GUS activity in the roots of germinating F_1 transformants

	No. of Seeds	No. of Germinated seeds	GUS assay Assayed fresh	Positive	%
Uncut stigma	113	112	89	13	14.6
Cut stigma	926	599	364	21	5.8

The histochemical assay described by Jefferson et al. (1987) was used to localize the appearance of the blue color of the X-Gluc cleavage product.

Example 4: Integration of NPTII[APH(3')II] gene into Wheat Genome:-Southern Blot Hybridization:

The presence of foreign DNA in the transgenic wheat, i.e. the aforesaid kanamycin resistant plants, was confirmed by Southern blotting as illustrated in Figs. 3-7 and 9. Total DNA (15 µg) extracted from leaves of the putative transformants and wild type plants, was digested by standard procedure with various restriction enzymes, separated on 0.8% agarose gels and then blotted onto nylon filters. The Apa I/Hind III fragment containing the entire NPT II gene (Fig. 2), isolated from pPCV702GUS and labelled

with ^{32}P was used as a probe for detecting the presence of the integrated foreign DNA in the wheat genome. Hybridization with the probe was carried out under stringent conditions, namely x5 Denhardts solution, x4 SSC, 0.1% SDS at 60°C and followed by four rinses in x4 SSC, 0.1% SDS at the same temperature.

As illustrated in Fig. 3a, total DNA was extracted from the wild type wheat cultivars Genaro 81 (VEERY #3"S", CIMMYT, Mexico), Seri 82 (VEERY #5"S", CIMMYT, Mexico) and Shafir (SON64A/TZPP//NAI60/3/FA) and from putative transformants expressing kanamycin resistance (Km^R), i.e. APTII activity, designated TAU89104–3 and TAU89107–1 which were transformed by plasmid pPCV702*nif*D. All the extracted DNAs were digested with the restriction endonucleases BamHI and HindIII and then separated on an agarose gel and transferred onto a nylon filter, by the above–noted standard procedures. As a positive control, two DNA samples from plasmid pPCV702GUS were also prepared, one digested with the enzymes BamHI and HindIII, the other with the enzymes ApaI and HindIII, these samples being separated on same above–noted agarose gel and transferred onto the nylon filter. The DNA samples on the nylon filter were hybridized with the aforesaid NPTII ApaI/HindIII fragment probe by the aforesaid hybridization procedure. The hybridization results as shown in Fig. 3a demonstrate that the transformant TAU89107–1 carries, on a high molecular weight BamHI/HindIII DNA fragment of about 10.5 kb, a foreign DNA which hybridizes with the NPTII probe. None of the wild type DNA fragments are capable of hybridizing to the NPTII probe, indicating that this gene is not present in these genomes. The schematic restriction map shown in Fig. 3b illustrates the possible mode of insertion of a part of the plasmid pPCV702*nif*D (Fig. 1) into the wheat plant genome, "genomic" denoting this genomic DNA. This map was constructed on the basis of the hybridization results shown in Fig. 3a and Fig. 4a and also on

the basis of the known restriction enzyme sites of the plasmid pPCV702*nif*D (Fig. 1), and the absence of *nif*D sequence in the DNA of the transformant TAU89107-1.

As shown in Fig. 4a, total DNA was extracted from the same wild type cultivars and transformant wheat plants as described for Fig. 3a, but these DNA samples were digested with the restriction endonuclease PstI only, which cuts each of the two plasmid vectors in the NPTII gene and in the amp^R gene. The digested DNA samples were separated on an agarose gel and were subsequently transferred to a nylon filter and hybridized as before with the NPTII ApaI/HindIII DNA fragment probe. The results of the hybridization reveal that transformant TAU89107-1 has two PstI DNA fragments (approx. 6.9 and 0.8 kb) which are capable of hybridizing said probe. The schematic restriction map shown on Fig. 4b) is the same as that of Fig. 3b) and was constructed on the basis of the hybridization results shown in Figs. 3a) and 4a) and on the basis of the known restriction sites of pPCV702*nif*D (Fig. 1).

As shown in Fig. 5, total DNA was extracted from the wild type wheat cultivar Shafir and from nine F_2 transformants of TAU89107-1 F_1 plants (1, 2, 3, 4, 5, 6, 7, 8 and 9), (see Figs. 3a and 4a). These DNA samples were digested with the restriction endonucleases PstI and BamHI, were separated on an agarose gel and transferred to a nylon filter as before. The DNA samples on the nylon filter were hybridized with the probe NPTII ApaI/HindIII DNA fragment + pPCV702*nif*D vector as noted above (Figs. 3a, 4a) under the aforesaid hybridization conditions. The results indicate that second generation transformants 4,5,6,8, and 9, in particular, carry PstI/BamHI DNA fragments (approx. 2.5 and 3.5 kb) which are capable of hybridizing to the probe and which appears to be absent from the wild type genomic DNA.

Example 5: Southern Hybridization of F_2 DNA

The presence of 10.5 kb fragment in the F_2 plants of TAU89107-1 transformants digested with BamHI and HindIII is shown in Fig. 6. Lanes 1 and 2 represent the F_2 transformant TAU89107-1-J18; lane 4 the F_2 TAU89107-1-K10 transformant derived from selfing of F_1 plants of TAU89-107-1; and lanes 3 and 5 represent the controls being DNA from the plasmid pPCV702nifD digested with EcoRI. The Southern blot procedures were as described above in Example 4. The probes used in these analyses were the following: the HindIII-ApaI NPTII fragment or the PCR synthesized gene 4 terminator (described in Example 8), both of which resulted in identical hybridization patterns.

Example 6: Southern Hybridization of F_3 DNA

The presence of 9.8kb and 10.5kb fragments in the F_3 plants of TAU89107-1 transformants digested with BamHI and HindIII is shown in Fig. 7. DNA from wild type Shafir is shown in lane 1. Lanes 2, 3 and 4 represent the following F_3 families: TAU89107-1-H29-21, TAU89107-1-J17-14 and TAU89107-1-J18-9, respectively. The Southern blot procedures were as described abve in Example 4. The probes used in these analyses, were those noted above in Example 5, namely, the HindIII-ApaI fragment or the PCR synthesized gene 4 terminator fragment, both of which resulted in identical hybridization patterns.

Example 7: PCR Amplification

In Fig. 8(a) there is shown a representation of the electrophoresis on an agarose gel, under standard conditions, of the DNA fragments obtained after the PCR procedure, namely, the results of the PCR amplification of the inserted NPTII coding sequence in F_2, F_3 and F_4 generation plants of sub-families of the transformant Shafir TAU89107-

1Km^R (for the PCR procedures see also Example 11 below). The DNA templates used in the PCR procedure were as follows:

Lane No. 1 = control sample, no DNA template in reaction;

Lane No. 2 = control sample, non-transformed, wild-type Shafir DNA;

Lane No. 3 = transformant sample, F_2 generation, Shafir TAU89107-1-B19 DNA;

Lane No. 4 = transformant sample, F_2 generation, Shafir TAU89107-1-J20 DNA;

Lane No. 5 = transformant sample, F_3 generation, Shafir TAU89107-1-J18-X DNA;

Lane No. 6 = transformant sample, F_3 generation, Shafir TAU89107-1-J18-9 DNA;

Lane No. 7 = transformant sample, F_4 generation, Shafir TAU89107-1-J18-9-1B DNA;

Lane No. 8 = transformant sample, F_4 generation, Shafir TAU89107-1-J18-9-1K DNA; and

Lane No. 9 = size markers for DNA fragments from ØX 174 bacteriophage DNA cleaved with HaeIII restriction endonuclease.

Thus, the presence of foreign DNA in sub-families of transformant Shafir TAU89107-1Km^R (see also Table 2 below, Example 11) in F_2, F_3 and F_4 generations was positively confirmed by direct PCR amplification as shown in Fig. 8(a). In all such transformants carrying the NPTII coding sequence, a 790 bp fragment was synthesized from the templates regardless of generation (F_2, F_3, F_4).

Figure 8(b) shows the results of the PCR amplification of the APH(3')II gene and its adjacent gene 4 terminator using total DNA extracted

from the F_4 progenies of transformant TAU89107-1Km^R obtained by selfing. The primers used in the PCR reactions were as follows:

Primer No. 1 corresponds to nucleotide 21 until the 47th nucleotide of the NPTII coding region (26 nucleotides) as follows:

= 5'-GCACGCAGGTTCTCCGGCCGCTTGGG-3'

Primer No. 2 corresponds to nucleotide 744 until nucleotide 775 of the NPTII coding region (31 nucleotides) as follows:

= 5'-CCCGATTCGCAGCGCATCGCCTTCTATCGCC-3'

Primer No. 3 is a reverse primer and represents the 3' end of gene 4 terminator as follows:

= 5'-ATTATACATAACACGCACA-3' (a total of 19 nucleotides).

The DNA templates and the primers used in the PCR amplification were as follows:

Lane No. 1 = size markers being bacteriophage λ DNA digested with BstEII;

Lane No. 2 = control template non-transformed wild-type Shafir DNA with primers 1 and 3;

Lane No. 3 = transformant sample, F_4 generation, Shafir TAU89107-1-H29-21-1A2 with primers 1 and 3 (about 1.15 kb);

Lane No. 4 = transformant sample, F_4 generation, Shafir TAU89107-1-J18-9-1B with primers 1 and 3;

Lane No. 5 = transformant sample, F_4 generation, Shafir TAU89107-1-J18-9-1B with primers 2 and 3 (about 0.5 kb);

Lane No. 6 = transformant sample, F_4 generation, Shafir TAU89107-1-J18-X-1B with primers 1 and 3;

Lane No. 7 = transformant sample, F_4 generation Shafir TAU89107-1-J18-X-1B with primers 2 and 3;

Lane No. 8 = Bacteriophage ϕx174 DNA digested with HaeIII.

The linkage between the NPTII coding region and the adjacent gene 4 terminator was maintained throughout 4 generations of selfing of the TAU89107-1Km^R family.

Example 8: Stability of the NPTII sequence

The stability of the NPTII sequence in different plant organs was evaluated in various F_4 transformants of the TAU89107-1.

The presence of a 10.5 kb fragment was confirmed in F_4 plants as shown by Southern blot analysis of representative plants of 3 families (Fig. 9). Total DNA extracted from the F_4 plants was digested with BamHI and HindIII and probed with the gene 4 (Poly A) terminator fragment which was obtained by PCR amplification (Figs. 1 and 2). The DNA templates were as follows:

Lane No. 1 = control template, non-transformed wild-type Shafir DNA.

Lane No. 2 = transformant sample, F_4 generation, Shafir TAU89107-1-H29-21-1A1C (C = refers to the central tiller from which DNA was extracted).

Lane No. 3 = transformant sample, F_4 generation Shafir TAU89107-1-H29-21-1A2C (a central tiller form another plant originated from the same F_3 spike as in Lane No. 2).

Lane No. 4 = transformant sample, F_4 generation Shafir TAU89107-1-J18-9-1BC (a central tiller from which DNA was extracted).

Lane No. 5 = transformant sample, F_4 generation Shafir TAU89107-1-J18-9-1BR (R = refers to the roots of the plant from which DNA was extracted).

Lane No. 6 = transformant sample, F_4 generation Shafir TAU89107-1-J18-X-1BC (a central tiller from which DNA was extracted).

Lane No. 7 = transformant sample, F_4 generation Shafir TAU89107-1-J18-X-1BR (roots of the plant from which DNA was extracted).

The presence in F_4 plants of the 10.5 kb fragment as shown by the results presented in Fig. 8b, should also contain the terminator (the gene 4 poly A sequence) linked to the NPTII coding region. Therefore the Southern blot analysis performed with the terminator sequence confirmed that the introduced sequence was stably maintained throughout 4 generations of selfing. The presence of two adjacent bands of about 10 kb was observed in F_3 and F_4 generations.

Example 9: Kanamycin-Resistant F_5 Plants

Resistance to kanamycin was expressed in seedlings of F_5 plants germinated on 150 µg/ml kanamycin. A representative F_5 plant is shown in Fig. 10a together with kanamycin-sensitive seedling of the wild-type Shafir. The F_5 plant (CC44) is a progeny of the TAU89107-1-J18-9 F_3 family. The mature F_5 kanamycin-resistant Shafir transformant plant resembles phenotypically (plant height, leaf and spike shape) the non-transformed wild-type Shafir. This representative F_5 plant was examined for

resistance to kanamycin at the seedling stage in F_1, F_3 and the F_5 generations, whereas seed multiplication via selfing without kanamycin was performed in the F_2 and F_4 generations.

The presence of the NPTII coding "tail" (51bp) and the linkage to the gene 4 (poly A) terminator was confirmed by PCR amplification using primers 2 and 3 of Fig. 8b (Example 7). The PCR product of the kanamycin-resistant transformant CC44 and the kanamycin-sensitive Shafir were electrophoresed on 1.2% agarose gel along with bacteriophage ϕx174 DNA digested with HaeIII (Fig. 10b). The DNA templates and the primers used in the PCR amplification were as follows:

Lane No. 1 = bacteriophage ϕx174 DNA digested with HaeIII.

Lane No. 2 = control template, non-transformed wild-type Shafir DNA with primers 2 and 3.

Lane No. 3 = transformant template extracted from kanamycin-resistant F_5 Shafir TAU89107-1-J18-9-1B-4A plant (= CC44) primed with primers 2 and 3 (about 0.5 kb).

Example 10: Integration of the GUS Reporter Gene

The presence of another foreign DNA sequence introduced by the application of the vector pPCV702GUS was confirmed in F_1 plants by Southern hybridization. The results are presented in Fig. 11 which shows total DNA extracted from F_1 wheat plants transformed with pPCV702GUS and probed with the same plasmid. In this Southern blot the Shafir (lane No. 1) and the transformant DNA samples (lanes 2 and 3) were about 5 µg of total genomic DNA. The samples were digested with the restriction endonucleases EcoRI and HindIII.

In order to confirm the presence of 2.9 kb which includes: 35S promotor-GUS coding region -NOS terminator, in the integrated DNA,

about 10 ng of the vector pPCV702GUS were digested with the same enzymes and used as a control sample in the Southern blot analysis (denoted as 702GUS).

Lane No. 1 = represents the wild-type Shafir control,

Lane No. 2 = represents the F_1 wheat transformant Seri 82 TAU90-56-13, and

Lane No. 3 = represents the F_1 transformant Pavon 76 TAU90-84-7.

The above observed results are evidence of the presence of the introduced 2.9 kb GUS fragment in F_1 transformants of the high-yielding commercial wheat cultivars Pavon 76 and Seri 82.

In a similar fashion, the F_1 sub-families of Seri 82 transformants TAU90 GUS/NPTII (see Table 2 below, Example 11, listed as transformants TAU9055-9 and TAU9056-13, transformed by the pPCV702GUS vector) were evaluated for the presence of GUS, NPTII or both by direct PCR as shown in Fig. 12. This figure shows a representation of the electrophoresis on an agarose gel, under standard conditions, of the above DNA fragments obtained after the PCR procedure, namely, the results of the PCR amplification of the inserted NPTII or GUS coding sequences in several F_1 generation sub-families of Seri 82 TAU90 GUS/NPTII transformants. The to DNA templates used in the PCR procedure and the amounts thereof were as follows:

Lane No.1 = control sample, GUS primers (primers Nos. 3 and 4, see Example 11, Table 2) added to reaction but no DNA template added;

Lane No. 2 = control sample, NPTII primers (primers Nos. 1 and 2, see Example 11, Table 2) added to reaction but no DNA template added;

Lane No. 3 = control sample, GUS primers and Seri 82 wild type DNA as template added (500 ng DNA);

Lane No. 4 = control sample, NPTII primers and wild type Seri 82 DNA as template added (500 ng DNA);

Lane No. 5 = transformant sample, GUS primers and TAU9055-9 DNA as template added (50 ng DNA);

Lane No. 6 = transformant sample, NPTII primers and TAU9055-9 DNA as template added (50 ng DNA);

Lane No. 7 = transformant sample, GUS primers and TAU9054-10 DNA as template added (50 ng DNA);

Lane No. 8 = transformant sample, GUS primers and TAU9054-11 DNA as template added (50 ng DNA) (no synthesis observed);

Lane No. 9 = transformant sample, NPTII primers and TAU9056-13 DNA as template added (200 ng DNA); and

Lane No. 10 = size markers for DNA fragments from ϕx174 bacteriophage DNA cleaved with HaeIII restriction endonuclease.

It should be noted that all the above transformants are part of the transformants presented in Table 2 (Example 11).

Thus, as shown in Fig. 12, 1170 bp GUS and 790 bp NPTII fragments were synthesized by the transformant TAU9055-9 (lanes 5 and 6, respectively). A GUS fragment was also confirmed in the TAU9054-10 transformant (lane 7), but not in the TAU9054-11 transformant (lane 8). An NPTII coding sequence insertion was also confirmed in the TAU9056-13 transformant (lane 9). This transformant was also represented in Fig. 11, where the 2.9 kb GUS fragment was observed.

All transformants that were verified by Southern blot and PCR amplification gave a positive GUS expression in the root phloem (Table 1).

Example 11: Transformations of different wheat varieties by two plasmid vectors

The high-yielding, semi-dwarf, spring bread wheat (*T. aestivum L.*) cultivars Genaro 81 (VEERY#3"S", CIMMYT, Mexico), KEA"S" (CM21335, CIMMYT, Mexico), Papago M86 (CM52359, CIMMYT, Mexico), Pavon 76(CM8399, CIMMYT, Mexico), Seri 82 (VEERY#5"S", CIMMYT, Mexico) and Shafir (SON64A/TZPP//NAI60/3/FA, Hazera Seed Co., Israel) (see also Examples 2-4 above) were examined for their transformational capability by way of administered foreign DNA. The above wheat strains were transformed using the procedures described in Example 2 above, the foreign DNA being administered to the plants being that detailed in Example 1 above. Following the transformation procedure, the treated plants were tested to determine the success and frequency of transformation using the bioassay methods described in Example 3 above and by a PCR assay carried out as follows:

In the transformants containing the NPTII coding sequence, the NPTII fragment was synthesized by PCR using total transformant DNA as template and the following primers:

Primer No. 1 (5' region of NPTII coding sequence):

5'-GCACGCAGGTTCTCCGGCCGCTTGGG-3'

Primer No. 2 (a reverse primer of the 3' region of NPTII coding sequence):

5'-GAAGGCGAGCGCTGCGAATCGGG-3'

In the transformants containing the GUS coding sequence, the GUS fragment was synthesized by PCR using total transformant DNA as template and the following primers:

Primer No. 3 (5' region of GUS):

5'-GTGGAATTGATCAGCGTTGGTGGG-3'

Primer No. 4 (a reverse primer of the 3' region of GUS):

5'-GCCAGTGGCGAAATATTCCCGTGC-3'

Transformation events occurring in different wheat cultivars during 2 cycles of experiments characterized by the above two assays are summarized in Table 2.

TABLE 2

Transformation events occurring in several spring wheat cultivars following application of plasmids carrying NPTII or GUS/NPTII genes (see Example 1)

Transformant Wheat	Wheat genotype	Introduced gene	Presence and expression	Transformation frequency*
TAU89104-8	Seri 82	NPTII	Km^R, $NPTII^{PCR}$	3.2% Km^R (1/31)
TAU89107-1	Shafir	NPTII	Km^R, $NPTII^{PCR}$	4.3% Km^R (1/23)
TAU89147-3	Genaro 81	GUS/NPTII	Km^R, $NPTII^{PCR}$	4.5% Km^R (1/22)
TAU89173-1	KEA "S"/Seri 82	GUS/NPTII	GUS^{PCR} Km^R, $NPTII^{PCR}$	4.3% Km^R (3/69)
TAU9055-9	Seri 82	GUS/NPTII	GUS+, $NPTII^{PCR}$ GUS^{PCR}	15.5% GUS^+ (20/129)
TAU9056-13	Seri 82	GUS/NPTII	GUS^+, $NPTII^{PCR}$	15.5% GUS^+ (20/129)
TAU9085-5	Pavon 76	GUS/NPTII	GUS^+, $NPTII^{PCR}$ GUS^{PCR}	2.2% GUS^+ (4/178)
TAU90115-9	Papago M86	GUS/NPTII	GUS^+, $NPTII^{PCR}$	4.7% GUS^+ (6/128)

In Table 2 above the various designations under "Presence and expression", denote the following:

Km^R = Transformant is capable of expressing the NPTII gene, i.e. is kanamycin resistant, as observed from green seedlings capable of growing on water agar containing 150 µg/ml kanamycin (Km) (see Example 3 above).

$NPTII^{PCR}$ = Presence of NPTII fragment detected directly in the transformant by use of PCR methods (see PCR procedure hereinbelow).

GUS^+ = Transformant exhibits GUS activity in the roots, as observed in a bioassay using X-Gluc as substrate (see Example 3 above).

GUS^{PCR} = Presence of GUS fragment detected directly in the transformant by use of PCR methods (see PCR procedure hereinbelow).

* = Evaluated according to kanamycin resistance (NPTII) in seedlings or GUS expression (GUS) in the roots.

Example 12: Integration of the bar gene into two different wheat varieties

The high yielding, semi-dwarf, spring bread wheat cultivars Seri 82 (VEERY #5"S", CIMMYT, Mexico) and Shafir (SON64A/TZPP//NAI60/3/FA, Hazera Seed Co., Israel) were transformed with the plasmid pPAT-NPTII 100.1-28 carrying the Streptomyces hygroscopicus *bar* gene (DeBlock et al., 1987, White et al., 1990). This gene encodes an acetyl CoA transferase capable of inactivating the herbicides bialaphos and glufosinate by acetylation. The gene was linked to the CaMV 35S promoter and PolyA (terminator) sequences to serve as a marker gene

in plants. The plasmid pPAT-NPTII 100.1-28 is a pUC-derivative (Topfer et al., unpublished, personal communication) which also carries the NPTII gene with the first intron of the maize *Shrunken* gene (sh1) (Werr et al., 1985) inserted downstream to the NPTII translational start site (Christoph, Maas and J. Schell, personal communication).

The *bar* sequence was detected by PCR amplification in transformants of the F_1 generation as shown in Figure 13, which is a representation of the electrophoresis on an agarose gel, under standard conditions of the 0.6 kb PCR product obtained, when specific *bar* primers were used. The first one corresponds to the nucleotide -8 until 19th nucleotide of the *bar* coding region (White et al., 1990) as follows:

** ****
CCGATCCCATATGAGCCCAGAACGACGCC

The translation start site is underlined.

Asterisks denote nucleotide substitutions to form the NdeI and BamHI sites and additional two deoxycytidines that were added to the 5' end.

The second primer is a reverse primer complementary to the sequence of nucleotide 533 to nucleotide 548 of the coding region, two stop signals and 16 nucleotides downstream according to White et al., (1990) and additional two deoxycytidines as follows:

5'-CCGGATCCCCCGGGTCATCAGATCTCGGTGACGGGC (37 mer)

With reference to Figure 13, the DNA templates used in the PCR procedure were the following:

Lane No. 1 = transformant sample, F_1 generation, TAU92P4SHF9–2, reamplified fragment after gel purification of the 0.6 kb fragment synthesized by PCR;

Lane No. 2 = transformant sample, F_1 generation TAU92P4SER14–1;

Lane No. 3 = transformant sample, F_1 generation TAU92P4SER14–1, reamplified fragment after gel purification of the 0.6 kb fragment obtained in the first PCR synthesis, (demonstrated in Lane 2);

Lane No. 4 = control sample, non–transformed, wild type Shafir DNA;

Lane No. 5 = control sample, non–transformed, wild type Seri 82 DNA;

Lane No. 6 = control sample, no template added in PCR reaction;

Lane No. 7 = control sample, plasmid pPAT–NPTII 101.1–28 as a template (30 ng); and

Lane No. 8 = size markers for DNA fragments from ϕx174 bacteriophage DNA cleaved with HaeIII.

The above–noted direct detection of the NPTII, GUS and bar gene fragments in the transformants, as set forth in Examples 7–12, was carried out using standard PCR procedures (Cetus Corp./Hoffmann–La Roche AG).

The present findings as set forth herein–above in Examples 3–12 and their accompanying Figures, support integration events of foreign DNA into the wheat genome by the method of the present invention. The frequencies of insertion were rather high ranging from 2.6 to 4.3% according to DNA probing and up to 15.5% according to GUS assays over two different years, for the NPTII and GUS/NPTII genes.

Integration and expression were shown for three different genes having two different promoters and terminators, and for different wheat cultivars of diverse genetical background. The wheat cultivars used

in the study were all high-yielding, modern commercial spring wheats, some of which are grown over large acreages in developed and developing countries. The phenology and fertility of most transformants matched that of the original cultivars regardless of generation (F_1 to F_5).

Thus, it may be concluded that by the method of the present invention it is possible to prepare transgenic wheat plants, in particular, those of commercial agronomic varieties which have stably integrated the foreign DNA and can transmit this DNA to the next generation.

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CLAIMS:

1. A method for producing transgenic wheat plants comprising:

(a) emasculating wheat plant florets by removing all pollinator anthers prior to their maturation and then inducing synchronized pollination by pollinating the stigmas of said florets with mature male pollinator anthers from the same or a different variety of wheat plant;

(b) applying a droplet of an aqueous DNA solution onto one or more pollinated stigmas, following a time period after pollination in which the pollen tubes in the pollinated stigmas have started to grow into the style but have not yet reached the ovary, said DNA solution comprising a suitable DNA vector carrying at least one gene foreign to the plant and capable of inducing the expression of a desirable trait in the plant and optionally an additional marker gene;

(c) maintaining said DNA solution droplet on said stigma in a humid environment for a period of time to ensure that said DNA vector reaches and enters the ovule;

(d) protecting the treated plants of step (c) from additional pollination by any nearby plants, growing said plants and collecting the seeds developed in said florets; and

(e) growing said seeds under conditions adapted for the selection of transformed wheat plants.

2. The method according to claim 1, wherein said pollinated stigma, to which said droplet of an aqueous DNA solution is added, is an intact stigma.

3. The method according to claim 2, wherein said DNA solution is added to said stigma about 1–2 hours after pollination, and said DNA solution is maintained in contact with said stigma for about 12–14 hours.

4. The method according to claim 1, wherein the tips of the pollinated stigmas are truncated after a suitable period of time to ensure that the growing pollen tubes are within the styles but before the ovary, said DNA solution being applied onto the truncated stimgas.

5. The method of Claim 4, wherein the truncation of the tips of the pollinated stigmas is carried out about ½–2½ hrs. after pollination and said droplet of DNA solution is maintained in contact with said cut stigma tips for about 12–14 hrs.

6. The method of Claim 5, wherein the truncation of the stigma tips is carried out about 1–2 hrs. after pollination.

7. The method of any one of Claims 1–6, wherein said DNA vector comprises a nitrogen fixation structural gene.

8. The method of Claim 7, wherein said gene is the Klebsiella pneumoniae nifD gene.

9. The method of any one of Claims 1–6, wherein said DNA vector comprises a reporter gene, which serves as an indication of successful transformation.

10. The method of Claim 9, wherein said reporter gene encodes β–glucuronidase.

11. The method of any one of Claims 1–6, wherein said DNA vector comprises a gene sequence capable of conferring herbicide resistance when expressed in transformed plants.

12. The method of Claim 11, wherein said gene sequence is one encoding the bar gene.

13. The method of any one of Claims 1–12, wherein said DNA vector also comprises a selectable marker gene.

14. The method of Claim 13, wherein said marker gene is one capable of conferring antibiotic resistance.

15. The method of Claim 14, wherein said marker gene is the APH(3')II (NPTII) gene which encodes aminoglycoside phosphotransferase conferring kanamycin resistance when expressed, said gene being linked to the nopaline synthase (NOS) promoter and to the TL-DNA ipt gene (gene 4) polyadenylation signal sequence acting as a terminator.

16. The method according to any one of Claims 1-15, wherein said vector DNA is plasmid pPCV702*nif*D having a restriction map as shown in Fig. 1 and comprising the coding region of Klebsiella pneumoniae nifD linked to a CaMV 35S promoter and a NOS terminator, and said APH(3')II (NPTII) gene with its NOS promoter and TL-DNA gene 4 terminator.

17. The method according to any one of Claims 1-15 wherein said vector DNA is plasmid pPCV702GUS having a restriction map as shown in Fig. 2 and comprising the coding region of the β-glucuronidase (GUS) gene linked to a CaMV 35S promoter and a NOS terminator and said APH(3')II (NPTII) gene with its NOS promoter and TL-DNA gene 4 terminator.

18. A DNA vector useful for the production of transgenic wheat plants in accordance with any one of Claims 1-17, comprising a gene encoding the A subunit of nitrogenase, said gene being a nitrogen fixation structural gene.

19. A DNA vector according to Claim 18 wherein said gene is the *Klebsiella pneumoniae nif*D gene.

20. A DNA vector according to Claim 18 or 19, additionally comprising a reporter gene.

21. A DNA vector according to Claim 20 wherein said reporter gene encodes β-glucuronidase.

22. A DNA vector useful for the production of transgenic wheat plants in accordance with any one of Claims 1-17, comprising a DNA sequence that is capable of conferring herbicide resistance when expressed in transformed plants.

23. A DNA vector according to Claim 22 wherein said DNA sequence encodes the bar gene.

24. A DNA vector according to any one of Claims 18-23 also comprising a marker gene.

25. A DNA vector according to Claim 24 wherein said marker gene is one encoding a product capable of conferring antibiotic resistance.

26. A DNA vector according to Claim 25 wherein said marker gene is the APH(3')II (NPTII) gene which encodes aminoglycoside phosphotransferase which confers kanamycin resistance when expressed, said gene being linked to the nopaline synthase (NOS) promoter and to the T_L-DNA (gene 4) polyadenylation signal sequence acting as a terminator.

27. The plasmid pPCV702nifD having a restriction map as shown in Fig. 1.

28. The plasmid pPCV702GUS having a restriction map as shown in Fig. 2.

29. Transgenic wheat plants prepared by the method of any one of Claims 1-17.

30. Transgenic wheat plant transformed by a DNA vector according to any one of Claims 18-26.

31. Transgenic wheat plants transformed by the plasmid pCV702*nif*D which has a restriction map as shown in Fig. 1.

32. Transgenic wheat plants transformed by the plasmid pCV702GUS. which has a restriction map as shown in Fig. 2.

33. Transgenic wheat plants of a commercial crop variety.

34. Transgenic wheat plants according to claim 33 being of the species *T. aestivum*.

35. Transgenic wheat plants according to claim 33 selected from the group consisting of ATIR (CNO "S"/PJ62/4/GLL/3/TOB//JAR "S"/CRESPO, Hazera Seed Co., Israel), BAGULA "S" (CM 59123, CIMMYT, Mexico), CIANO 79 (CM31678, CIMMYT, Mexico), Genaro 81 (VEERY#3"S", CIMMYT, Mexico), KAUZ "S" (CM67458, CIMMYT,

Mexico), KEA"S" (CM21335, CIMMYT, Mexico), MILAN (23IBWSN37, CIMMYT, Mexico), OPATA M 85 (CM40038, CIMMYT, Mexico), Papago M86 (CM52359, CIMMYT, Mexico), PAVON 76 (CM8399, CIMMYT, Mexico), Seri 82 .(VEERY#5"S", CIMMYT, Mexico), and Shafir (SON64A/TZPP//NAI60/3/FA, Hazera Seed Co., Israel).

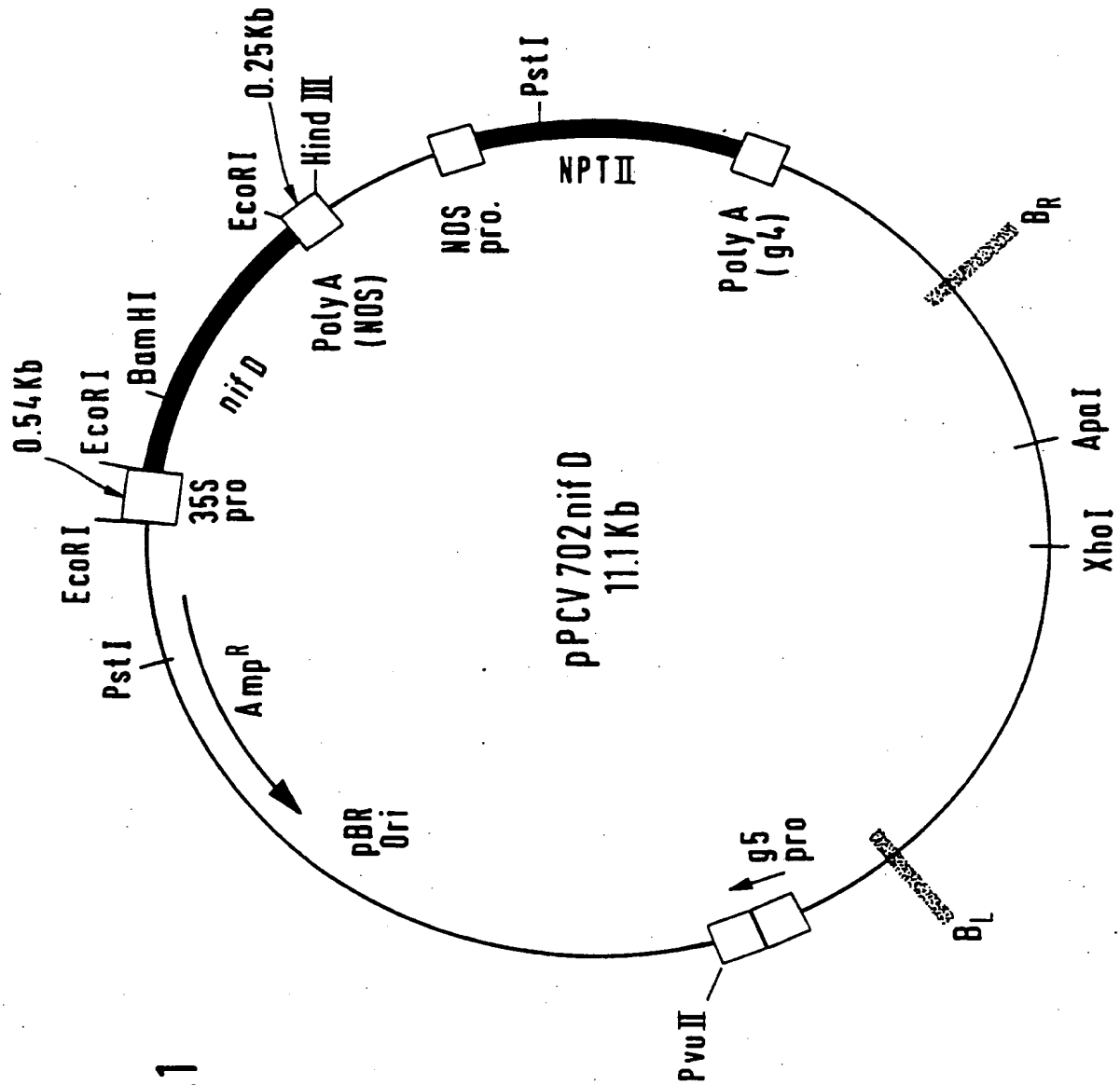

0.25Kb
Hind III
EcoRI
Pst I
NPTII
NOS pro.
Poly A (NOS)
Poly A (g4)
B_R
BamHI
EcoRI
0.54Kb
nifD
35S pro
ApaI
XhoI
EcoRI
Pst I
Amp^R
pPCV702nifD
11.1Kb
pBR Ori
g5 pro
B_L
Pvu II

Fig.1

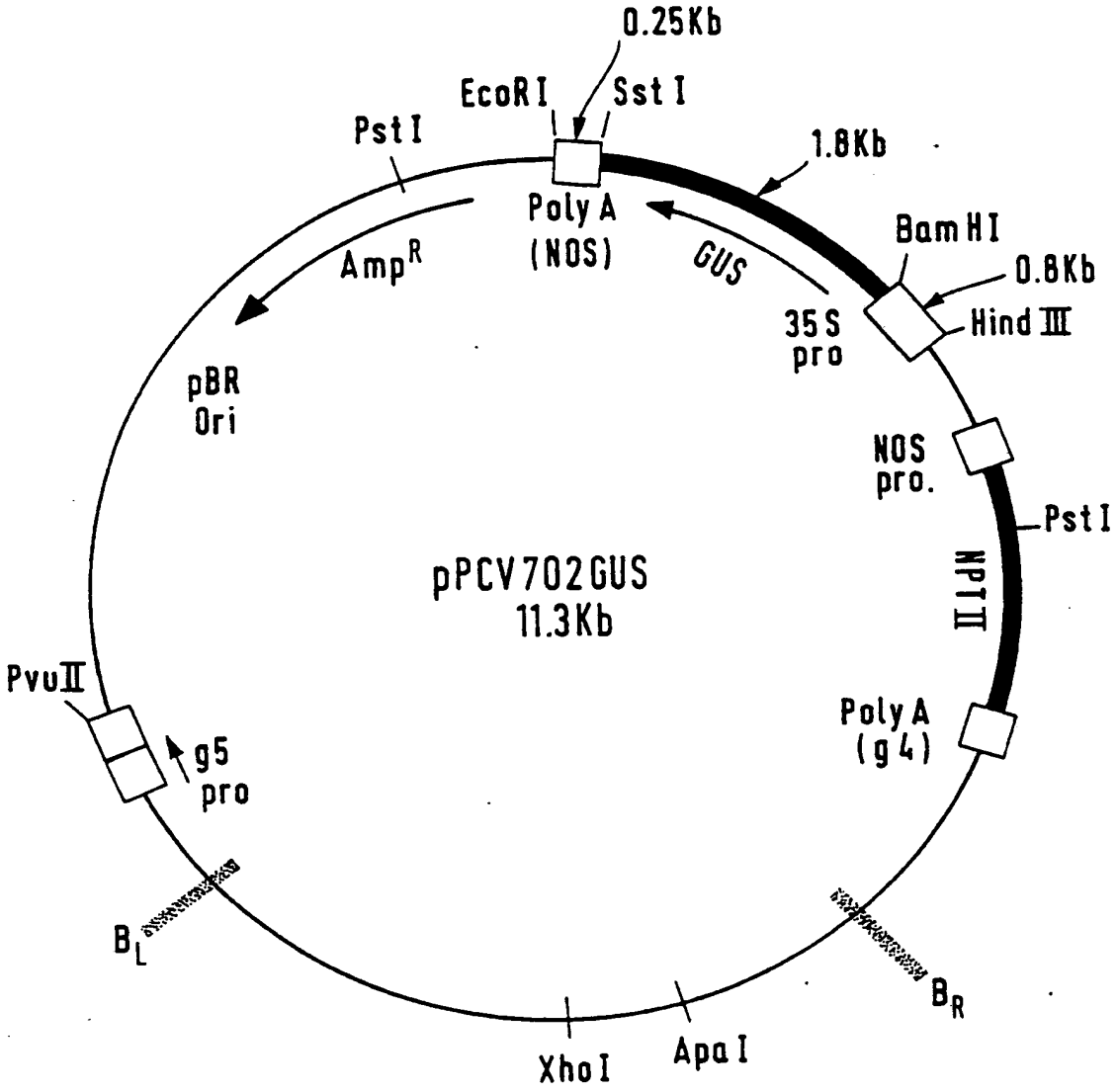

0.25Kb
EcoR I
Sst I
Pst I
1.8Kb
Poly A
(NOS)
Amp^R
GUS
Bam HI
0.8Kb
Hind III
35 S
pro
pBR
Ori
NOS
pro.
Pst I
pPCV702GUS
11.3Kb
NPTII
Pvu II
Poly A
(g 4)
g5
pro
B_L
B_R
Xho I
Apa I

Fig. 2

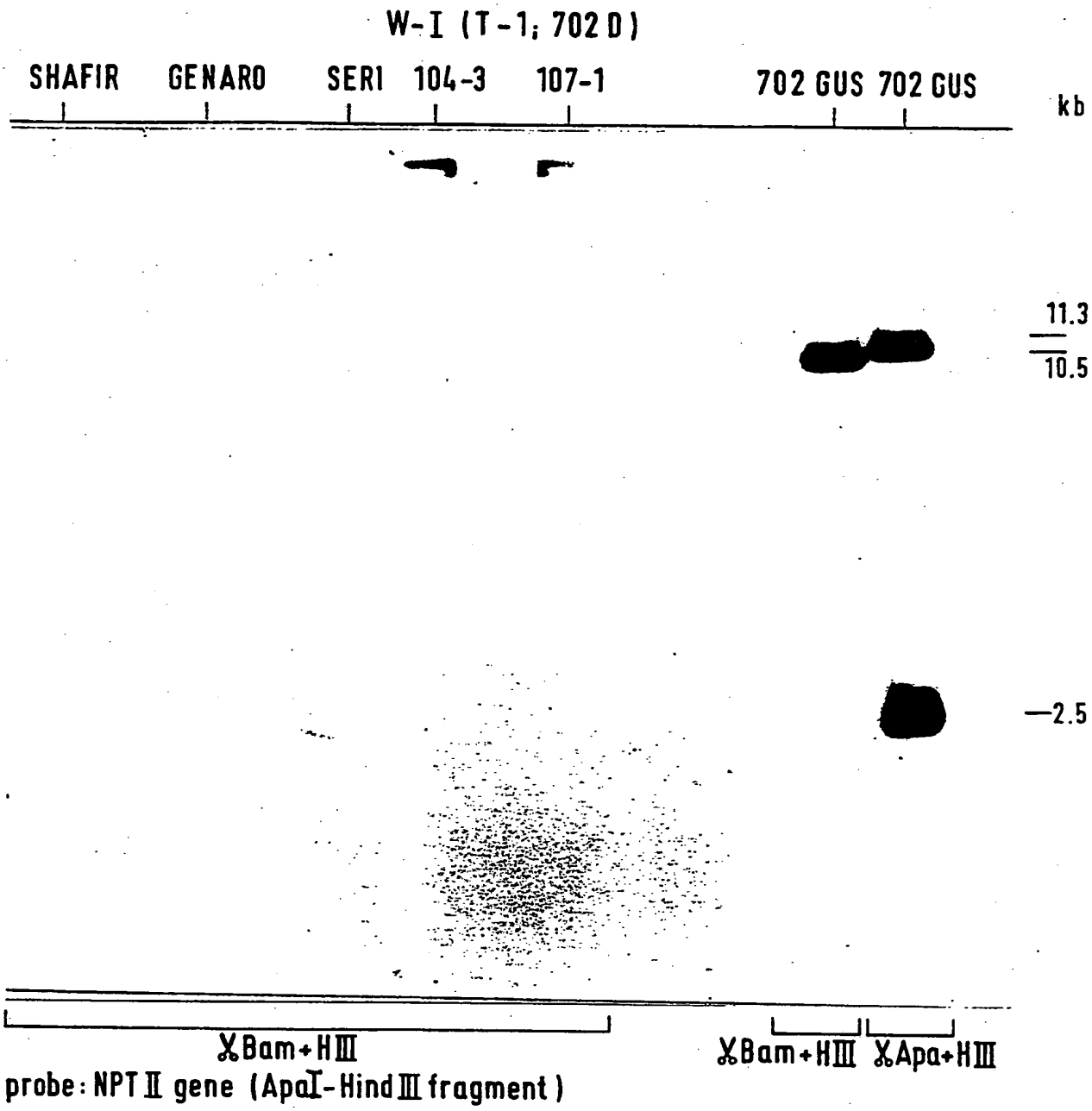

W-I (T-1; 702 D)
SHAFIR
GENARO
SERI
104-3
107-1
702 GUS
702 GUS
kb
11.3
10.5
2.5
XBam+HIII
XBam+HIII
XApa+HIII
probe: NPT II gene (ApaI-Hind III fragment)

Fig. 3a

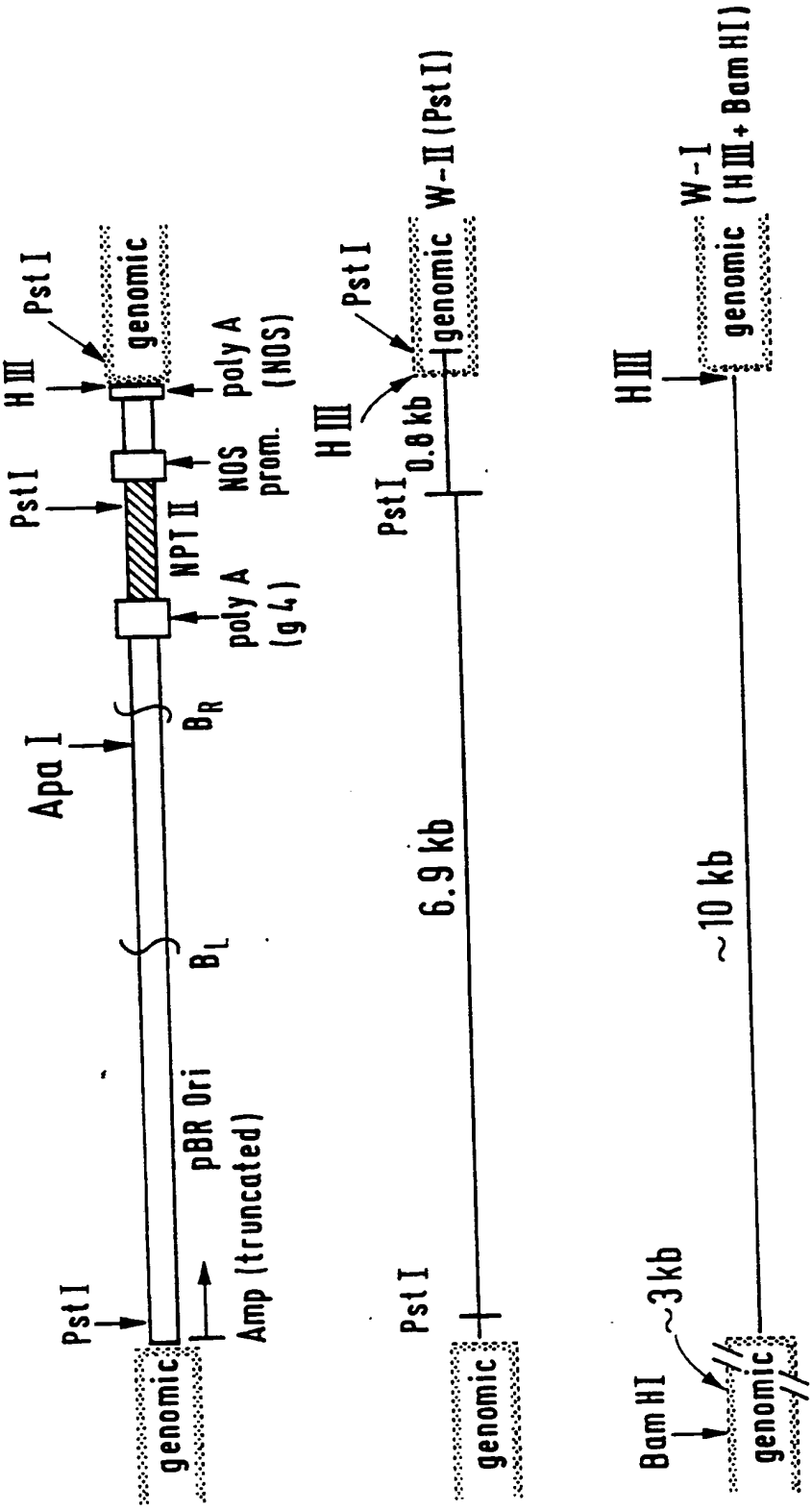

Pst I
genomic
H III
poly A (NOS)
NOS prom.
Pst I
NPT II
poly A (g 4)
Apa I
B_R
B_L
pBR Ori
Amp (truncated)
Pst I
genomic
H III
Pst I
genomic W-II (Pst II)
Pst I
0.8 kb
6.9 kb
Pst I
genomic
H III
W-1
genomic (H III + Bam HI)
~10 kb
Bam HI
~3 kb
genomic

Fig.3b

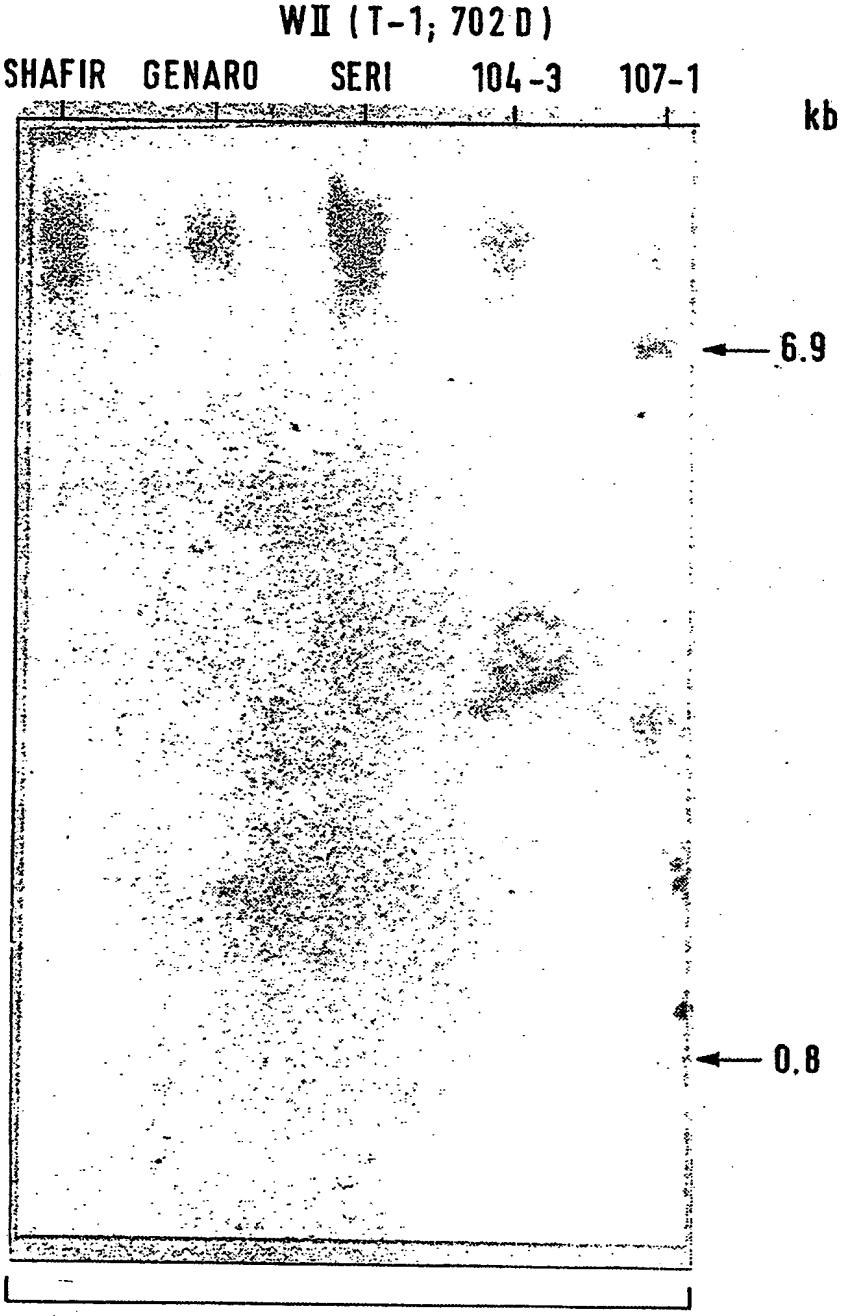

WII (T-1; 702 D)
SHAFIR GENARO SERI 104-3 107-1
kb
6.9
0.8
Pst I
probe: NPT II gene (ApaI-Hind III fragment)

Fig. 4a

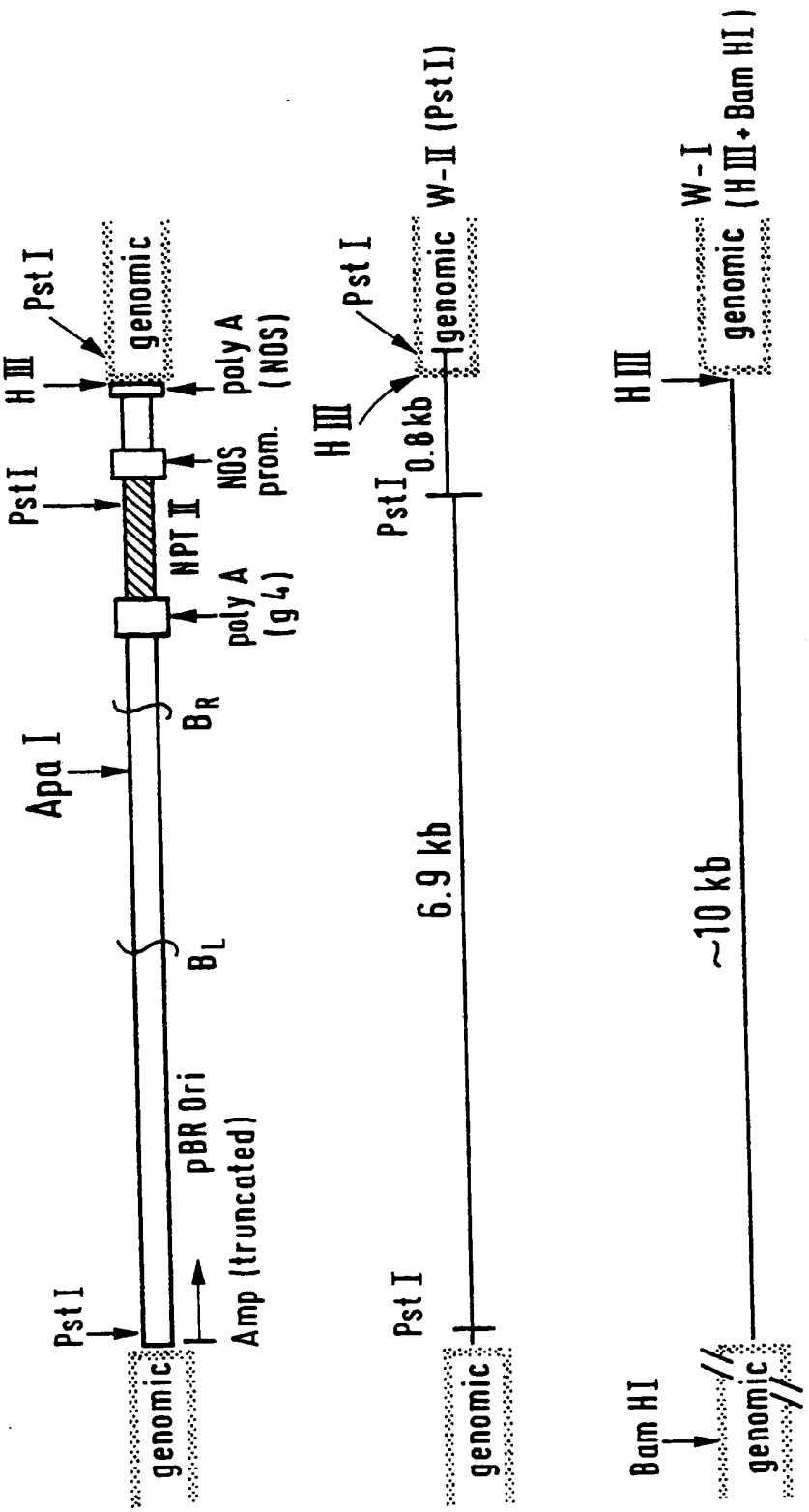
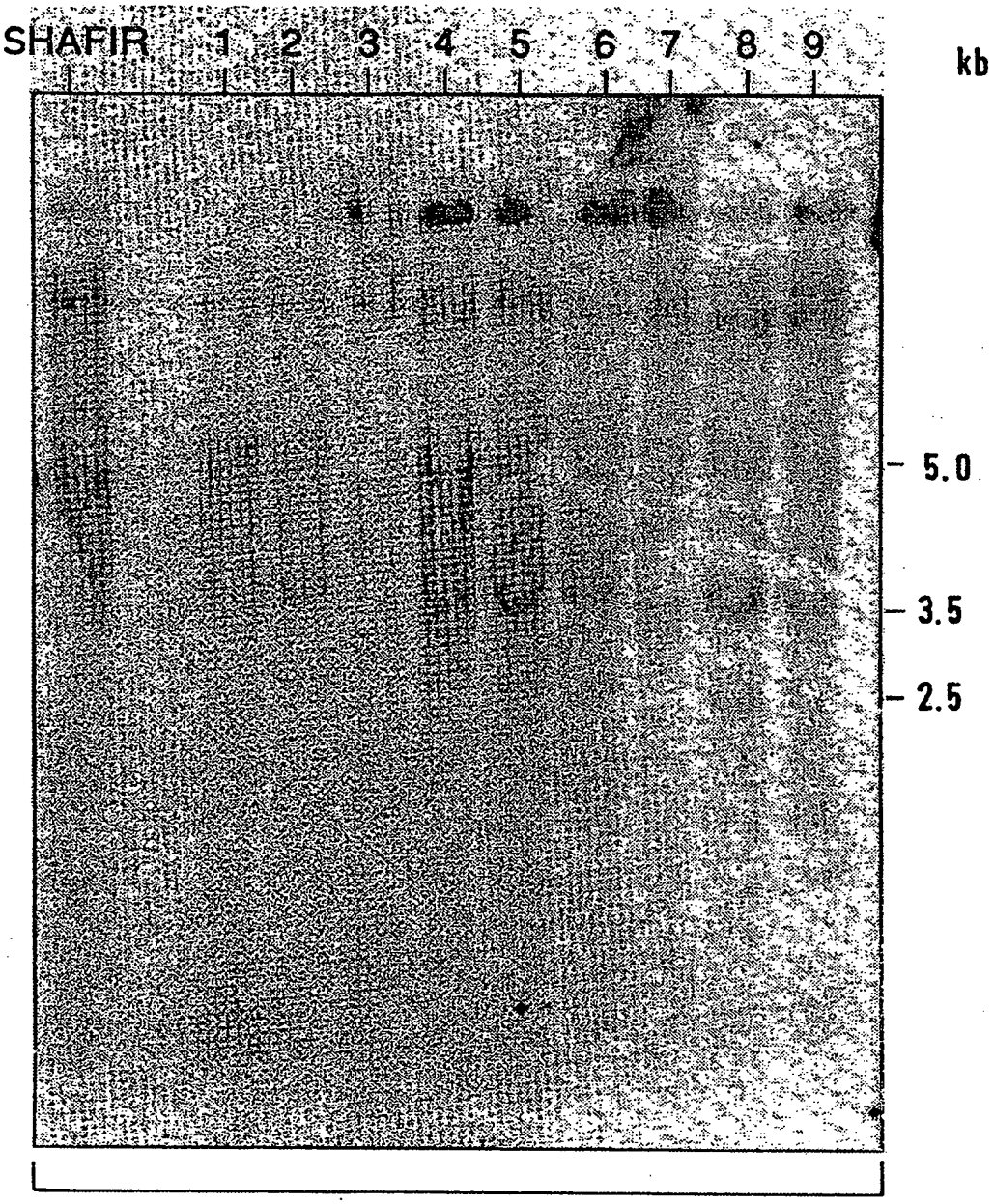

Pst I
H III
genomic
poly A (NOS)
NOS prom.
NPT II
poly A (g4)
Pst I
Apa I
B_R
B_L
pBR Ori
Amp (truncated)
Pst I
genomic
H III
Pst I
genomic W-II (Pst I)
Pst I 0.8kb
6.9 kb
Pst I
genomic
H III
W-I
genomic (H III + Bam HI)
~10 kb
Bam HI
genomic

Fig.4b

W-VII (T-2; selfing of 107-1, 702 D)



X PstI + BamHI

probe: NPT II fragment + 702 D vector

Fig. 5

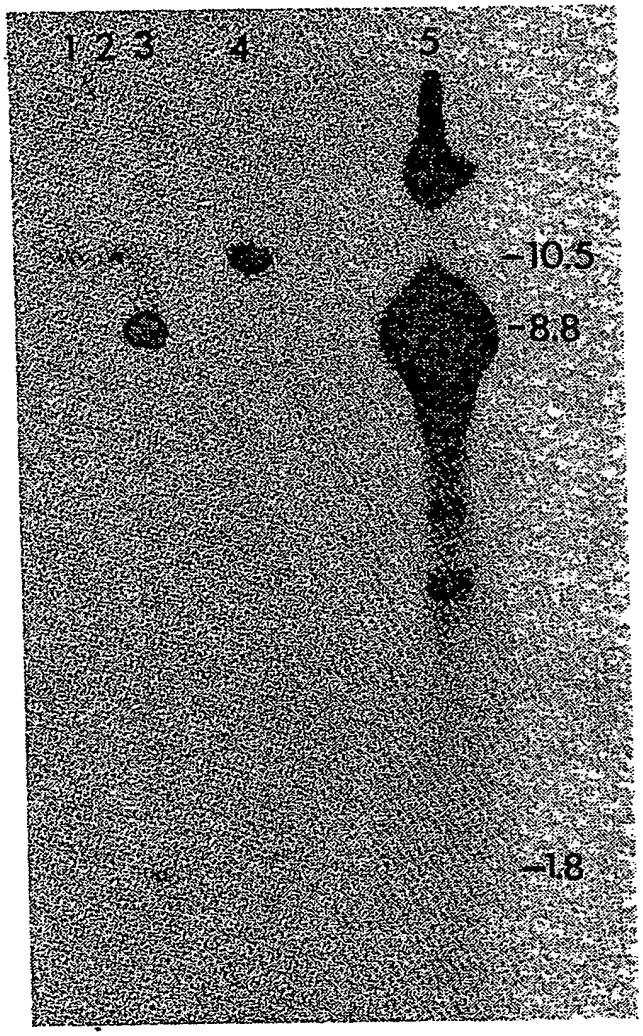


Fig. 6

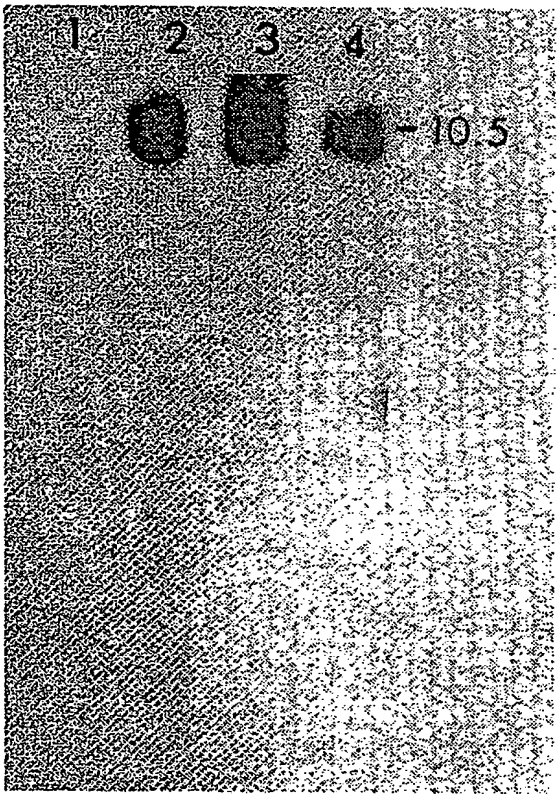


Fig. 7

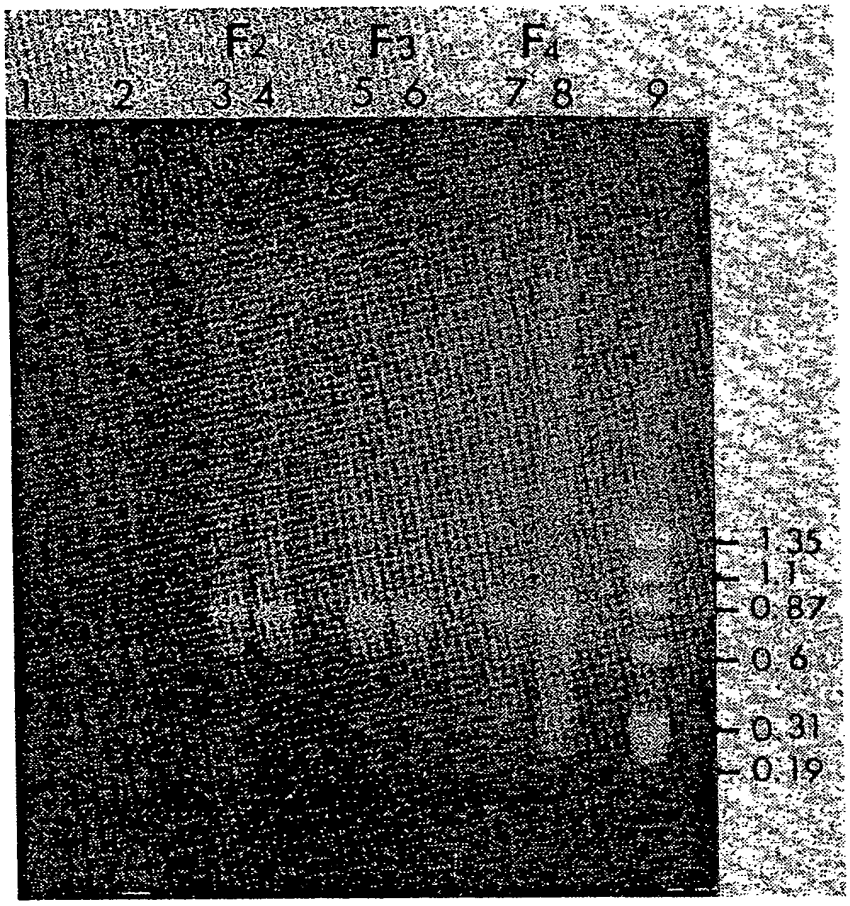


Fig. 8a

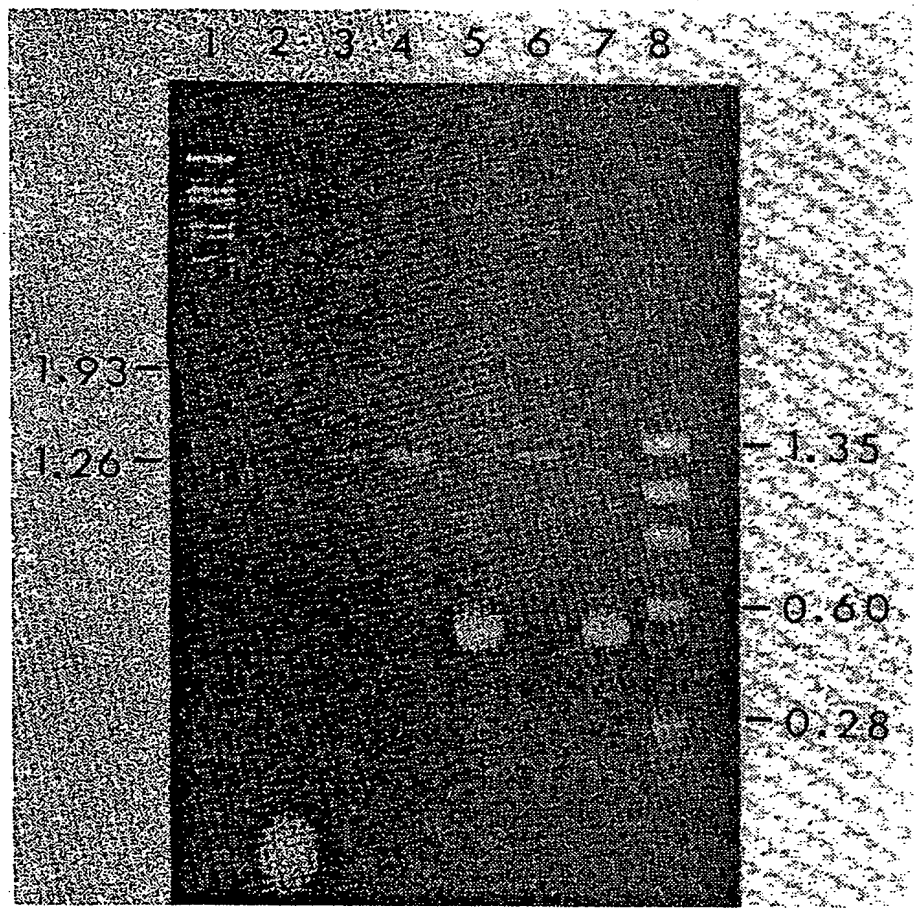


Fig. 8b

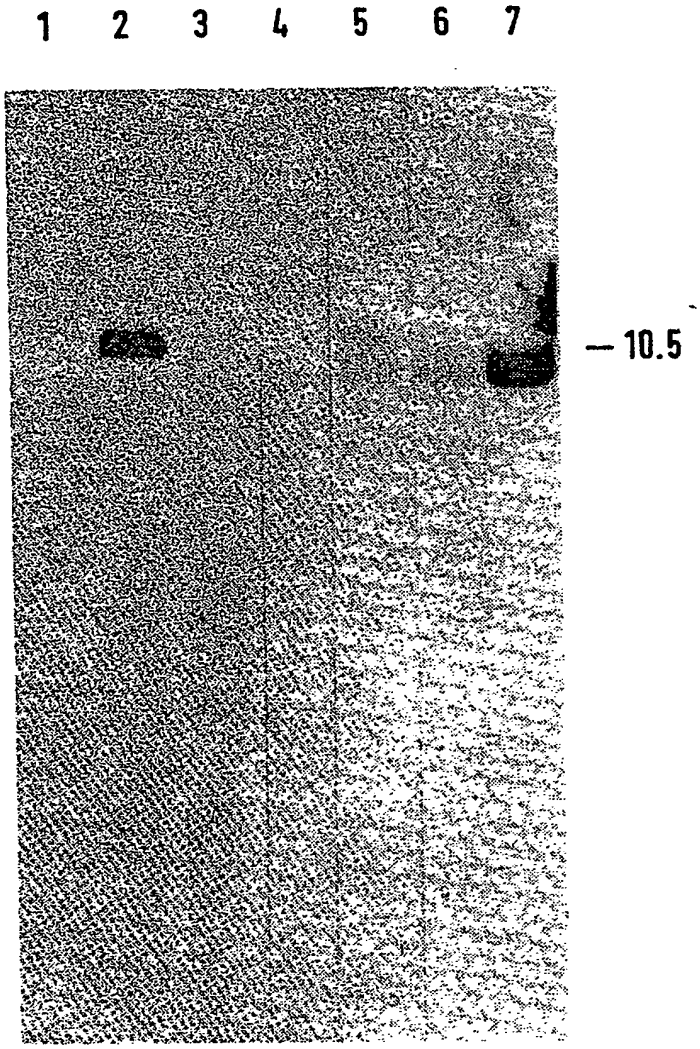


Fig. 9

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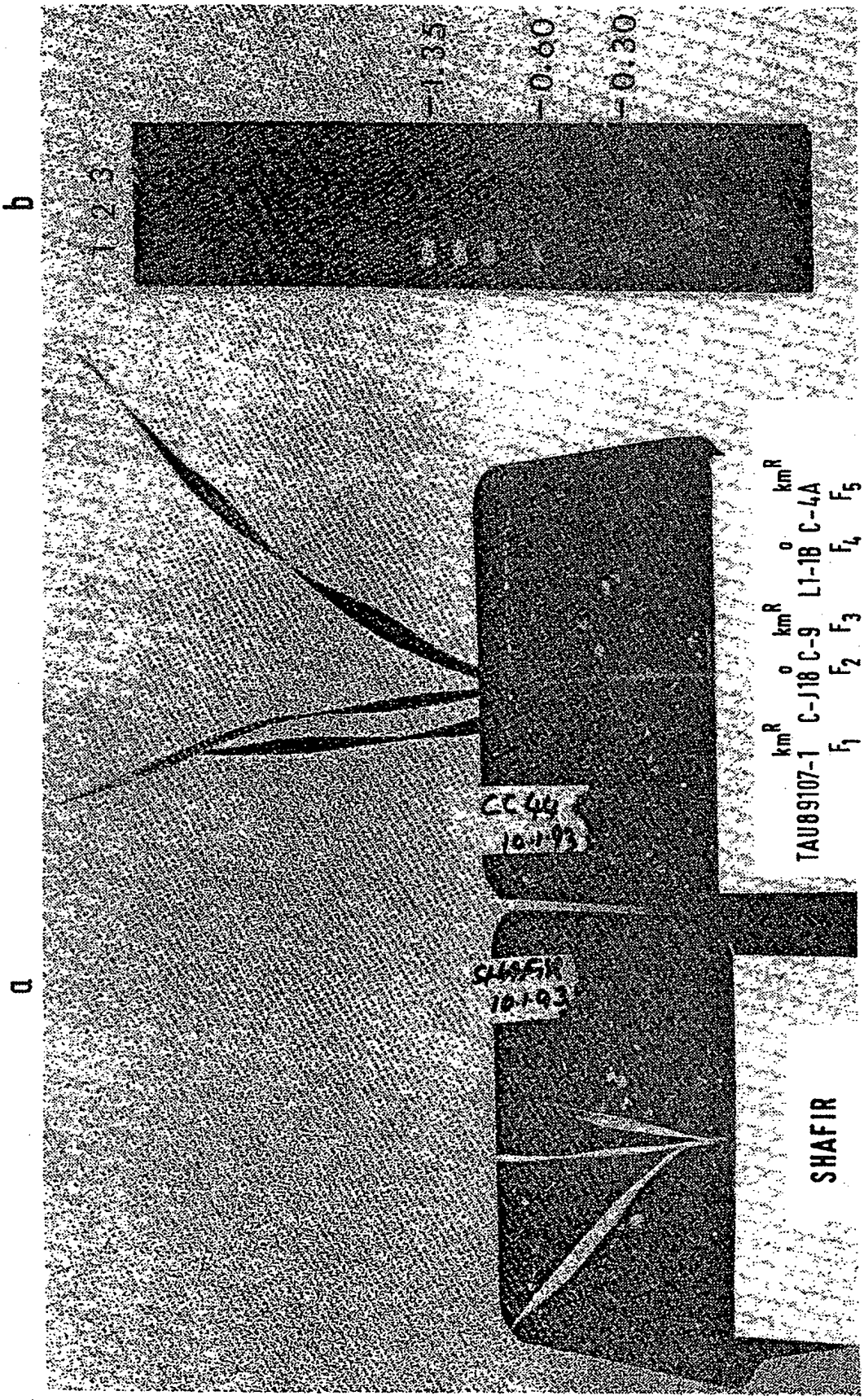
b
a
1 2 3
-1.35
-0.60
-0.30
SHAFIR
TAU89107-1 C-J18 C-9 L1-1B C-4A
F1 F2 F3 F4 F5
CC44
10.1.93
SHAFIR
10.1.93

Fig. 10

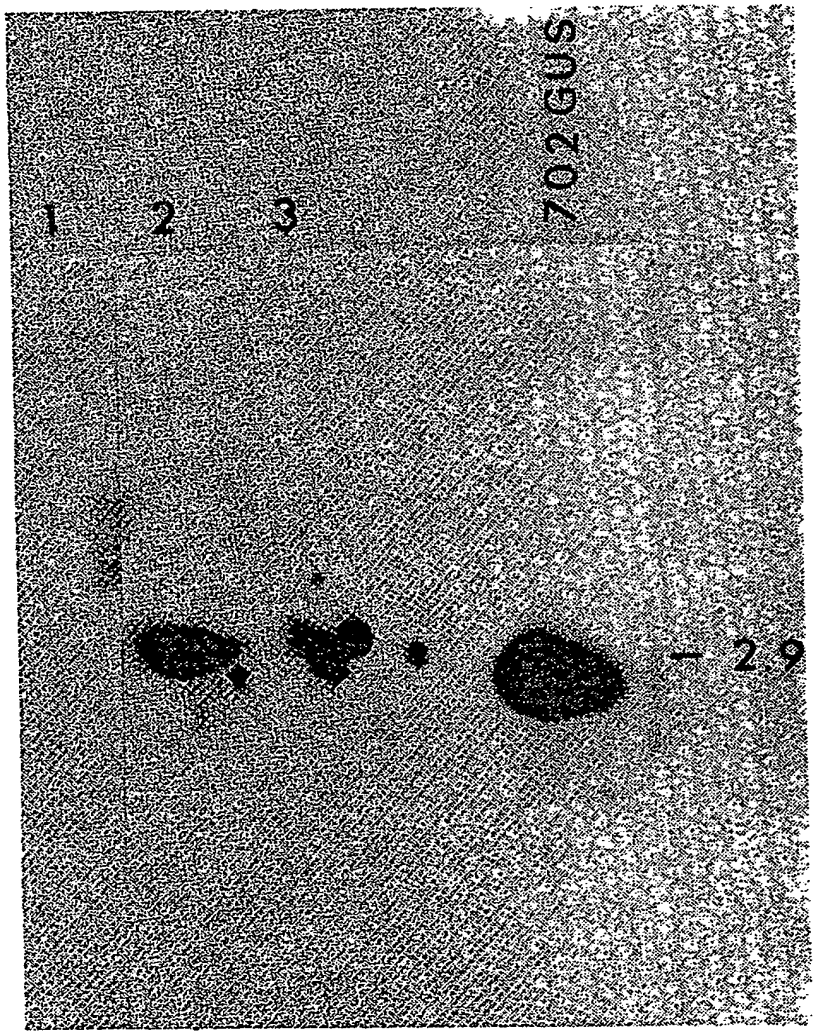

Fig. 11

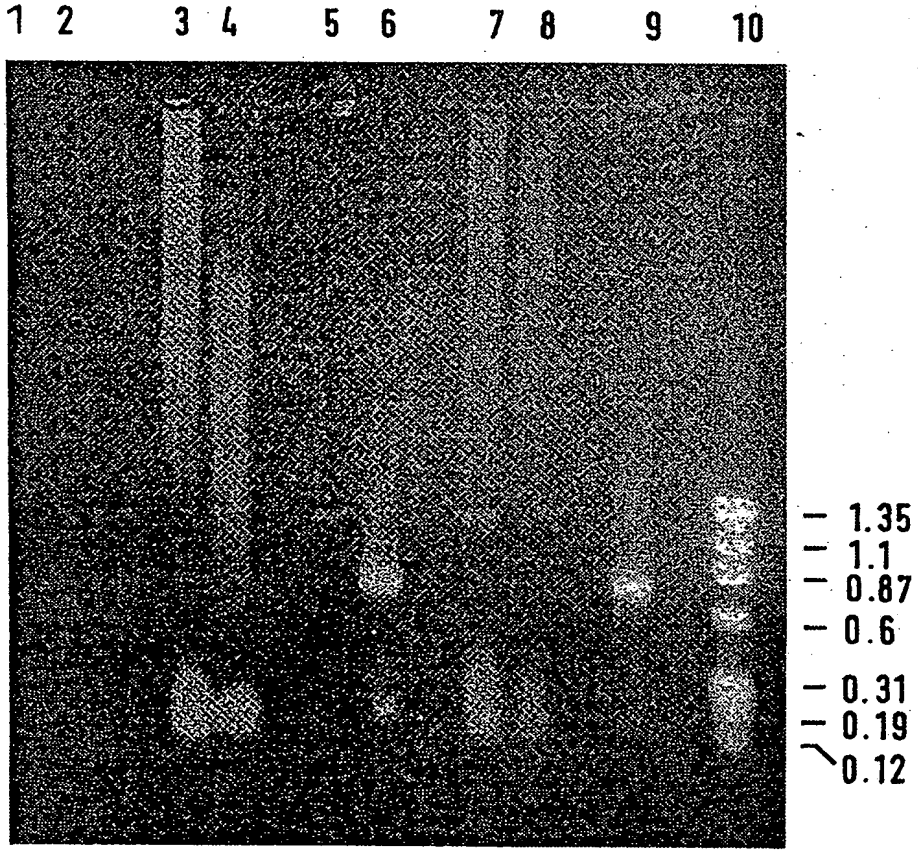

Fig. 12

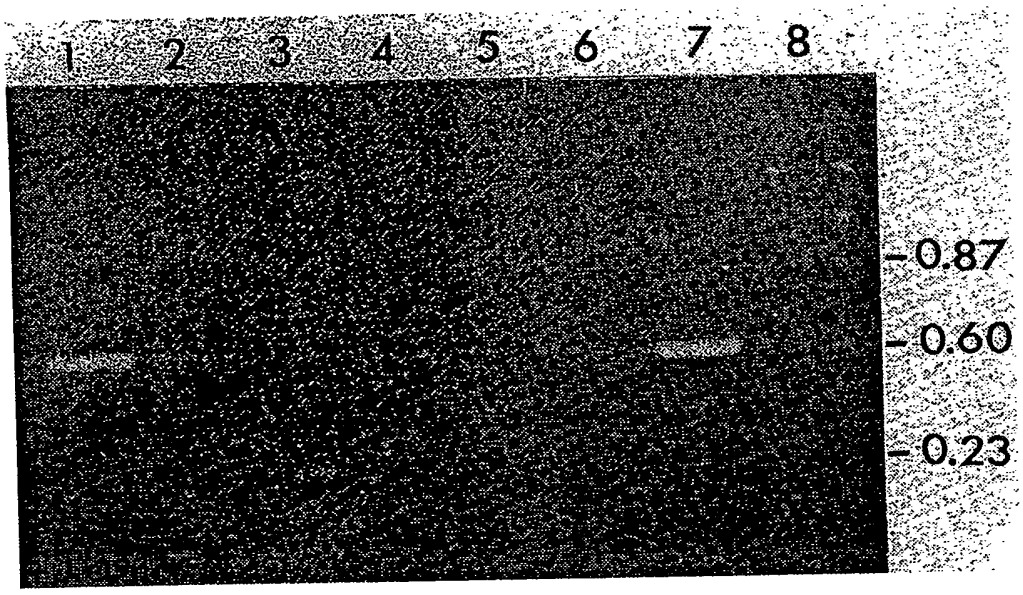

Fig. 13